

**PSEUDOTYPED RETROVIRUSES AND STABLE CELL LINES FOR  
THEIR PRODUCTION**

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**REFERENCE TO RELATED APPLICATIONS**

The present application claims the benefit of U.S. Patent Application Serial Number 60/095,242, filed on August 4, 1998, and U.S. Patent Application Serial Number 60/112,405, filed on December 15, 1998, which are both hereby incorporated by reference in their entirety.

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**BACKGROUND OF THE INVENTION**

The present invention relates generally to cells that produce pseudotyped retroviruses having broad host range. Specifically, the invention relates to cells that produce retroviruses pseudotyped with glycoproteins derived from either filoviruses or viruses having at least two different viral glycoproteins disposed in their lipid bilayer. The invention further relates to methods of producing such cells, the pseudotyped retroviruses produced, methods of making and using the pseudotyped retroviruses and kits for producing the pseudotyped retroviruses.

Retroviruses are ribonucleic acid (RNA) viruses that include an RNA genome enclosed within a viral capsid wherein the capsid is surrounded by an envelope, or lipid bilayer. Glycoproteins present in the lipid bilayer (envelope glycoproteins) interact with receptors on the surface of various host cells and allow the retroviruses to enter the host cell. Once in the cell, the retroviruses reverse transcribe the RNA of the viral genome into a double-stranded DNA (a proviral intermediate), and incorporate the deoxyribonucleic acid (DNA) into the cellular genome as a provirus. Gene products from the integrated foreign DNA may then be produced so that progeny viral particles may be assembled. As retroviruses can be modified to carry exogenous nucleotide sequences of interest, such recombinant retroviruses have a variety of uses. For example, such recombinant retroviruses are important in introducing desired exogenous

sequences into a cell, so that relatively high levels of the protein encoded by the sequences may be produced. However, use of such recombinant retroviruses has several drawbacks.

For example, retroviruses do not have a broad host range. Efforts at  
5 increasing the host range of retroviruses have included substituting the envelope glycoproteins of the virus with that of a different virus, thus forming a pseudotyped retrovirus. The pseudotyped retrovirus advantageously has the host range of the different virus. However, some retroviruses have been pseudotyped with viral glycoproteins that are toxic  
10 to cells, so the cells can only produce the virus for a limited time. Furthermore, in many cases, the pseudotyped retroviruses can not be stably produced and may not be produced at a high titer.

There is therefore a need for pseudotyped retroviruses of broad host range, and cell lines capable of producing such pseudotyped retroviruses.  
15 The present invention addresses this need.

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## SUMMARY OF THE INVENTION

It has been discovered that cells may be constructed to produce inventive retroviruses pseudotyped with viral glycoproteins, wherein the retroviruses have a broad host range. Accordingly, one aspect of the invention provides eukaryotic cells that include a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein. In one preferred embodiment, the fourth nucleotide sequence encodes at least two different viral glycoproteins, preferably togaviral glycoproteins, such as, for example, alphaviral glycoproteins. In alternative embodiments, the fourth nucleotide sequence encodes a filoviral glycoprotein, such as, for example, a Marburg virus or Ebola virus glycoprotein. In a preferred form of the invention, the cells stably produce inventive pseudotyped retroviruses.

A second aspect of the invention provides methods of forming the above-described eukaryotic cells. The method includes transfecting a eukaryotic cell with a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein. In one preferred embodiment, the fourth nucleotide sequence encodes at least two different viral glycoproteins, preferably togaviral glycoproteins. In alternative embodiments, the fourth nucleotide sequence encodes a filoviral glycoprotein, such as a Marburg virus glycoprotein. In preferred forms of the invention, the first, second, third and fourth nucleotide sequences are chromosomally-integrated, wherein the cell stably produces inventive pseudotyped retroviruses.

A third aspect of the invention provides inventive pseudotyped retroviruses, including a retroviral capsid, a lipid bilayer surrounding the retroviral capsid and at least one viral glycoprotein disposed in the lipid

bilayer. In inventive pseudotyped retroviruses, at least two different viral glycoproteins are disposed in the lipid bilayer, and in preferred embodiments, the viral glycoproteins are togaviral glycoproteins. In an alternative embodiment, the viral glycoprotein is a filoviral glycoprotein, preferably a Marburg virus glycoprotein.

In yet a fourth aspect of the present invention, methods of introducing nucleotide sequences into a cell are provided, and include transducing a cell permissive for viral entry with a pseudotyped retrovirus having a retroviral capsid, a lipid bilayer surrounding the retroviral capsid, at least one viral glycoprotein disposed in the lipid bilayer and a desired ribonucleotide sequence. In one preferred form of the invention, the cells are permissive for entry of a virus having at least two different viral glycoproteins in its lipid bilayer, such as a togavirus wherein the viral glycoproteins are togaviral glycoproteins. In alternative embodiments, the viral glycoprotein is a filoviral glycoprotein, preferably a Marburg virus glycoprotein.

A fifth aspect of the invention provides methods of screening agents effective in blocking viral entry into a cell. In one mode of practicing the invention, the method includes treating a pseudotyped retrovirus with the agent, treating a cell permissive for viral entry with the treated pseudotyped retrovirus and identifying eukaryotic cells having the desired marker. In one embodiment, the pseudotyped retrovirus has a retroviral capsid, a lipid bilayer surrounding the capsid, at least two different viral glycoproteins disposed in its lipid bilayer, such as togaviral glycoproteins wherein the cell is permissive for togaviral entry, and a nucleotide sequence encoding a desired marker. In alternative embodiments, a method is provided for screening agents effective in blocking filoviral entry, preferably Marburg virus entry, into a cell. Pseudotyped retroviruses having Marburg virus glycoprotein disposed in their lipid bilayer are preferred as are cells permissive for Marburg virus entry.

In yet another embodiment of a method of screening agents effective in blocking viral entry into a cell, the method includes treating a cell permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer with said agent, contacting the  
5 treated cell with a pseudotyped retrovirus having a retroviral capsid, a lipid bilayer that surrounds the retroviral capsid, at least two different viral glycoproteins disposed in its lipid bilayer, such as togaviral glycoproteins wherein the cell is permissive for togaviral entry, and a nucleotide sequence encoding a desired marker, and identifying cells having the  
10 marker. In alternative embodiments, a method is provided for screening agents effective in blocking filoviral entry, preferably Marburg virus entry, into a cell. Pseudotyped retroviruses having Marburg virus glycoprotein disposed in their lipid bilayer are preferred as are cells permissive for Marburg virus entry.

15 In a sixth aspect of the present invention, kits for forming inventive pseudotyped retroviruses are provided. The kits include a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide  
20 sequence encoding at least one viral glycoprotein. In one embodiment, the fourth nucleotide sequence encodes at least two viral glycoproteins, such as togaviral glycoproteins. In alternative embodiments, the fourth nucleotide sequence encodes a Marburg virus glycoprotein.

One object of the invention is to provide a eukaryotic cell including a  
25 first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein, such as a Marburg virus glycoprotein, preferably at least two viral glycoproteins, such  
30 as togaviral glycoproteins and especially alphaviral glycoproteins.

Another object is to provide a eukaryotic cell that includes a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth  
5 nucleotide sequence encoding at least one viral glycoprotein, such as a Marburg virus glycoprotein, preferably at least two viral glycoproteins, such as togaviral glycoproteins and especially alphaviral glycoproteins, wherein the cell stably produces the inventive pseudotyped retroviruses.

Another object is to provide a method of making the inventive cells  
10 described above, as well as the pseudotyped retroviruses so produced.

Other objects are to provide a method of screening agents effective in blocking either filoviral entry into a cell or entry of viruses having more than one viral glycoprotein in their lipid bilayer, such as togaviruses, and methods of introducing desired nucleotide sequences into a cell.

15 Yet other objects of the invention are to provide kits for forming inventive pseudotyped retroviruses.

These and other objects and advantages of the present invention will be apparent from the descriptions herein.

## BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows a Western blot of proteins derived from lysates of stable cell line SafeRRnslacZ, or precursor gpnslacZ cells, as further described in Example 4.

FIG. 2 depicts Giemsa solution-stained SafeRR-nslacZ cells (Panel A, FIG. 2A) and  $\Phi$ NX cells (Panel B, FIG. 2B) after being incubated at room temperature for one hour with pH 5.5 fusion buffer and grown in D-MEM FBS/PS culture medium for four hours as described in Example 5. Panel C (FIG. 2C) depicts Giemsa solution-stained SafeRR-nslacZ cells treated in a similar manner with the exception that they were exposed to pH 7 fusion buffer instead of pH 5.5 fusion buffer.

FIG. 3 depicts graphs showing the effects of lysosomotropic agents on transduction of the indicated retroviruses. Left panel, A, FIG. 3A, shows the effect of ammonium chloride and right panel, B, FIG. 3B, shows the effect of chloroquine. RRV, pseudotyped virus obtained from supernatants of SafeRR-nslacZ cells; Mo-MuLV, wild type Moloney murine leukemia virus expressing the env glycoprotein; VSV; Moloney murine leukemia virus pseudotyped with vesicular stomatitis viral glycoprotein G.

FIG. 4 shows fluorescence profiles of NIH 3T3 cells transduced with supernatant medium from  $\Phi$ NX cells (top panel, A, FIG. 4A) or Safe-Ebola-GFP cells (bottom panel, B, FIG. 4B) according to the procedure outlined in Example 9.

FIG. 5 depicts syncytia formation by packaging cells expressing Ebola glycoprotein. The cells were treated according to the protocol in Example 10. Top panel, A, (FIG. 5A) SafeEbola-GFP cells; Bottom panel, B, FIG. 5B,  $\Phi$ NX cells.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

For the purposes of promoting an understanding of the principles of the invention, reference will now be made to preferred embodiments and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations and further modifications of the invention, and such further applications of the principles of the invention as illustrated herein, being contemplated as would normally occur to one skilled in the art to which the invention relates.

The present invention relates to eukaryotic cells that stably produce pseudotyped retroviruses and methods for their production, pseudotyped retroviruses, methods of introducing nucleotide sequences into a target cell, methods of screening agents effective in blocking viral entry into cells and kits for forming inventive pseudotyped retroviruses.

It has been discovered that eukaryotic cells may be constructed that either transiently or stably produce pseudotyped retroviruses having at least two different viral glycoproteins disposed in their lipid bilayer, such as togaviral glycoproteins. It has further been discovered that eukaryotic cells may be constructed that stably produce pseudotyped retroviruses having filoviral glycoproteins disposed in their lipid bilayer. The pseudotyped retroviruses of the present invention are advantageous in transducing cells of interest, are not toxic to the cells, have a broad host range and do not allow for pseudotransduction (i.e., introduction of proteins and/or genetic material without stable transmission of genetic material). Moreover, the present disclosure is the first report of a pseudotyped retrovirus having two different viral glycoproteins, with different membrane spanning domains, disposed in its lipid bilayer.

Accordingly, one aspect of the invention provides inventive eukaryotic cells having nucleotide sequences encoding retroviral Gag polypeptide, retroviral Pro polypeptide, retroviral Pol polypeptide and at



least one viral glycoprotein, such as a filoviral glycoprotein, or at least two viral glycoproteins, such as togaviral glycoproteins. In a preferred embodiment, nucleotide sequences encoding the polypeptides described are chromosomally-integrated and thus stably produce inventive  
5 pseudotyped retroviruses. A second aspect of the invention provides methods of forming cells that produce inventive pseudotyped retroviruses. A third aspect of the invention provides the inventive pseudotyped retroviruses, preferably those that include at least two different viral glycoproteins disposed in their lipid bilayer, including togaviral  
10 glycoproteins, and further preferably those that include a desired nucleotide sequence in their genome. Other aspects of the invention provide inventive methods of introducing a nucleotide sequence into a desired cell and methods of screening agents effective in blocking viral entry into a target cell, preferably blocking entry of a Marburg virus, or a virus having more  
15 than one viral glycoprotein in its lipid bilayer such as a togavirus, wherein all of the methods utilize the inventive pseudotyped retroviruses and cells described above, and kits for producing inventive pseudotyped retroviruses.

As discussed above, one aspect of the invention provides eukaryotic  
20 cells, forming inventive eukaryotic cell lines, having nucleotide sequences encoding retroviral Gag polypeptide, retroviral Pro polypeptide, retroviral Pol polypeptide and at least one viral glycoprotein, such as a filoviral glycoprotein, or at least two different viral glycoproteins, typically from the same virus, such as togaviral glycoproteins. The term "eukaryotic cell line"  
25 as used herein is intended to refer to eukaryotic cells that are grown *in vitro*. The term "nucleotide sequence", as used herein, is intended to refer to a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, and derivatives thereof. The terms "encoding" and "coding" refer to the process by which a nucleotide sequence, through the  
30 mechanisms of transcription and translation, provides the information to a

cell from which a series of amino acids can be assembled into a specific amino acid sequence to produce a polypeptide.

In forming a cell that produces an inventive pseudotyped retrovirus, a wide variety of cells may be selected. Eukaryotic cells are preferred, 5 whereas mammalian cells are more preferred, and include human, simian canine, feline, equine and rodent cells. Human cells are most preferred. It is further preferred that the cell be able to reproduce indefinitely, and is therefore immortal. Examples of cells that may be advantageously used in the present invention include NIH 3T3 cells, COS cells, Madin-Darby 10 canine kidney cells and human embryonic 293T cells. However, highly transfectable cells, such as human embryonic kidney 293T cells, are preferred. By "highly transfectable" it is meant that at least about 50%, more preferably at least about 70% and most preferably at least about 80% of the cells can express the genes of the introduced DNA.

15 The retroviral *gag*, *pro* and *pol* nucleotide sequences, and other retroviral nucleotide sequences for forming the specified pseudotyped retroviruses, may be obtained from a wide variety of genera in the family Retroviridae, including, for example, Oncoviruses, including Oncovirus A, B, C and D, lentiviruses and spumavirus F. Such sequences are preferably 20 obtained from the Moloney murine leukemia virus (MMLV; in the genus Oncovirus C). Such sequences are well known in the art. For example, nucleotide sequences encoding MMLV *gag*, *pro* and *pol* may be found in Bereven et al., *Cell* (1981) 27:97-108. Most preferably, such sequences are obtained from lentiviruses. Unlike most retroviruses, lentiviruses have 25 the capacity to integrate the genetic material they carry into the chromosomes of non-dividing cells as well as dividing cells. Therefore, lentiviral nucleotide sequences encoding proteins that allow for chromosomal integration of virally transported nucleic acid in non-dividing cells are advantageously employed, as the host range of the pseudotyped 30 retroviruses will be broadened.

The above-described retroviruses are readily publicly available from the American Type Culture Collection (ATCC) and the desired nucleotide sequences may be obtained from these retroviruses by methods known to the skilled artisan. For example, the nucleotide sequences may be  
5 obtained by recombinant DNA technology. Briefly, viral DNA libraries may be constructed and the nucleotide sequences may be obtained by standard nucleic acid hybridization or polymerase chain reaction (PCR) procedures, using appropriate probes or primers. Alternatively, supernatant medium from cells infected with the respective virus can be isolated and the desired  
10 retroviral nucleotide sequences may be amplified by PCR. Such vectors may also be constructed by other methods known to the art.

It is preferred that the *gag*, *pro* and *pol* nucleotide sequences are contiguous to each other as found in native retroviral genomes, such as in the order 5'-*gag*-*pro*-*pol*-3'. It is further preferred that these retroviral  
15 nucleotide sequences are chromosomally-integrated into the cellular genome. Furthermore, the *gag*-*pro*-*pol* nucleotide sequences are operably linked at the 5' end of the *gag* nucleotide sequence to a promoter sequence, so that transcription of the sequences may be achieved.

A nucleic acid sequence is "operably linked" to another nucleic acid  
20 sequence, such as a promoter sequence, when it is placed in a specific functional relationship with the other nucleic acid sequence. The functional relationship between a promoter and a desired nucleic acid typically involves the nucleic acid and the promoter sequences being contiguous such that transcription of the nucleic acid sequence will be facilitated. Two  
25 nucleic acid sequences are further said to be operably linked if the nature of the linkage between the two sequences does not (1) result in the introduction of a frame-shift-mutation; (2) interfere with the ability of the promoter region sequence to direct the transcription of the desired nucleotide sequence, or (3) interfere with the ability of the desired  
30 nucleotide sequence to be transcribed by the promoter sequence region.

Typically, the promoter element is generally upstream (i.e., at the 5' end) of the nucleic acid coding sequence.

A wide variety of promoters are known in the art, including cell-specific promoters, inducible promoters, and constitutive promoters. The promoters may further be selected such that they require activation by activating elements known in the art, so that production of the protein encoded by the specified nucleic acid sequence may be regulated as desired. It is well within the purview of a person skilled in the art to select and use an appropriate promoter in accordance with the present invention. For example, the promoters that may be advantageously present in the cell, 5' to the gag-pro-pol sequences, include rat actin promoter and the MMLV promoter. Furthermore, the cytomegalovirus promoter has been found to be an excellent promoter in the inventive system.

Other regulatory elements, such as enhancer sequences, which cooperate with the promoter and transcriptional start site to achieve transcription of the nucleic acid insert coding sequence, may also be present in the cell 5' to the nucleotide sequences that encode retroviral proteins. By "enhancer" is meant nucleotide sequence elements which can stimulate promoter activity in a cell, such as a bacterial or eukaryotic host cell.

A wide variety of viral glycoproteins may be advantageously present in the inventive cells of the present invention, especially viral glycoproteins necessary for attachment of the virus to a target cell and penetration of the virus into the cytoplasm of the cell, as well as viral glycoproteins necessary for maturation of the glycoproteins necessary for attachment and penetration of the virus. For example, the cells described above may include nucleotide sequences encoding at least two different viral glycoproteins. Examples of such viruses include viruses in the families Togaviridae (e.g., in the genus *Alphavirus* or *Rubivirus*), Flaviviridae (e.g., *Flavivirus*, *Pestivirus* and *Hepatitis C*), Paramyxoviridae (e.g., *Morbillivirus*), and Bunyaviridae (e.g., *Hantavirus*). Such nucleotide sequences are well

known to the art. In one embodiment, the cells may include, instead of the viral nucleotide sequences encoding at least two different viral glycoproteins, nucleotide sequences encoding filoviral glycoproteins. Examples of such viruses include Ebola virus (including Ebola Zaire, Ebola Reston and Ebola Sudan sequences which are chromosomally-integrated), and Marburg virus. These nucleotide sequences may be obtained by methods known in the art as recited in example 2. For example, nucleotide sequences encoding particular glycoproteins may be isolated and cloned into plasmids by standard techniques, and the nucleotide sequence may then be amplified by PCR using the appropriate primers.

In one form of the present invention, the cells include nucleotide sequences encoding glycoproteins from an alphavirus. In a most preferred embodiment, the cells include nucleotide sequences encoding glycoproteins from the viral species Ross River (depicted in SEQ ID 1). The viral transmembrane glycoprotein complex that is responsible for the binding of the alphavirus to the surface of a susceptible cell and for the fusion of the viral and cellular membranes that occurs during the process of viral entry includes a trimer of a heterodimer of two transmembrane proteins, which are denoted  $E_1$  and  $E_2$  and which are encoded by an  $E_3$ - $E_2$ -6K- $E_1$  glycoprotein coding region ( $E_3$  and 6K refer to viral proteins involved in maturation of  $E_1$  and  $E_2$  as known in the art) on the alphaviral genome. The  $E_2$ - $E_1$  coding region includes an  $E_3$  glycoprotein coding region as well as the 6K protein coding region. Such nucleotide sequences may be obtained by methods known to the skilled artisan as discussed for the *gag*, *pro* and *pol* nucleotide sequences above. For example, the  $E_2$ - $E_1$  coding region may be obtained as discussed in Kuhn et al. (1991) *Virology* 182:430-441. The  $E_2$ - $E_1$  glycoprotein coding region is also operably linked to a promoter sequence, such as described above, at its 5' end.

The eukaryotic cells described above, that include nucleotide sequences encoding togaviral glycoproteins, advantageously produce retroviruses pseudotyped with togaviral glycoproteins at a titer of at least

about  $1 \times 10^3$  transforming units (TU)/ml of cell culture supernatant medium. The cells more preferably produce such retroviruses at a titer of at least about  $1 \times 10^5$  TU/ml of supernatant and most preferably at a titer of at least about  $1 \times 10^6$  TU/ml of supernatant.

5 It is expected that other viruses not specifically mentioned herein having at least two different glycoproteins of similar structure to the glycoproteins in the viral families denoted above may be advantageously used in the present invention.

In another embodiment, the cells include nucleotide sequences  
10 encoding glycoproteins from a filovirus. Such filoviruses also exhibit a broad host range. A wide variety of nucleotide sequences that encode filoviral glycoproteins may be used to produce the inventive cells of the present invention. For example, nucleotide sequences encoding  
15 glycoproteins from the Marburg and Ebola virus (in the family Filoviridae and, including, for example, Ebola-Zaire and Ebola-Reston) may be introduced into the cells described above to produce a pseudotyped retrovirus. SEQ ID 2 shows the Ebola Zaire glycoprotein-encoding sequence and SEQ ID 3 shows the Marburg virus glycoprotein-encoding sequence. The nucleotide sequences encoding the filoviral glycoproteins  
20 may be obtained as described in Sanchez et al. (1993) *Virus Res.* 29 (3):215-240 and Will et al., (1993) *J. Virol.* 67:1203-1210. Moreover, such sequences may be obtained by other methods known to those skilled in the art, as described above for the togaviruses.

Eukaryotic cells described above that include the filoviral nucleotide  
25 sequences advantageously produce retroviruses pseudotyped with a filoviral glycoprotein at a titer of at least about  $4.5 \times 10^4$  TU/ml of supernatant. The cells more preferably produce such retroviruses at a titer of at least about  $1 \times 10^6$  TU/ml of supernatant and most preferably at a titer of at least about  $1 \times 10^7$  TU/ml of supernatant.

It is expected that other viruses not specifically mentioned above and having glycoproteins of similar structure to the filoviral glycoproteins may be advantageously used in the present invention.

The cells may transiently produce the retrovirus pseudotyped with at  
5 least two different viral glycoproteins, such as togaviral glycoproteins, or with a filoviral glycoprotein, but preferably stably produce such retroviruses. In one preferred form of the present invention, the nucleotide sequences encoding either the filoviral glycoproteins or encoding at least two different viral glycoproteins (such as togaviral glycoproteins) in the eukaryotic cells  
10 are chromosomally-integrated, so that the cell stably produces the pseudotyped retrovirus. By "stably produce", it is meant that the cells will produce pseudotyped retrovirus indefinitely (i.e., during the life span of the cell). Conversely, by transient production, it is meant that the cells will produce pseudotyped retrovirus for a period of at least about 24 hours,  
15 more preferably at least about 48 hours, and most preferably at least about 72 hours.

In a further preferred form of the present invention, the eukaryotic cells described above may include another nucleotide sequence that encodes a desired protein so that they may produce pseudotyped  
20 retroviruses having an RNA genome including such desired nucleotide sequences. The protein can be such that it provides a beneficial or therapeutic effect if introduced into an animal. For example, a gene may encode a protein that is needed by an animal, either because the protein is no longer produced, is produced in insufficient quantities to be effective in  
25 performing its function, or is mutated such that it either no longer functions or is only partially active for its intended function. The nucleotide sequence may be introduced into the cellular genome in a variety of ways known to the skilled artisan. For example, defective retroviruses (i.e., those which do not have the capability to produce all of the viral proteins necessary for  
30 production of a retrovirus having the ability to infect a cell and produce progeny viruses) may be constructed to include such a sequence in their

RNA genome and can then transduce a cell. Alternatively, and as described above, plasmid vectors may be used to introduce the nucleotide sequence, preferably DNA, encoding the desired protein. In either case, the vector typically includes nucleotide sequences necessary for production of the pseudotyped retrovirus. For example, the RNA sequence in the viral genome is flanked on the 5' end by a splice acceptor site and a splice donor site followed by a sequence necessary for packing of the viral genome (such as a psi sequence) and a long terminal repeat (LTR), all as known in the art. The 3' end of the RNA sequence may be flanked on its 3' end with a polypurine tract followed by another LTR, further as known to the skilled artisan. The vectors may include other nucleotide sequences known to the art that are necessary for transduction.

In one preferred form, the desired protein may be one that allows entry of the virus into a cell to be detected. For example, a visually detectable component, or marker, such as one that emits visible wavelengths of light, or that may be reacted with a substrate to produce color of specified wavelengths. For example, such nucleotide sequences include the nucleotide sequence encoding the *Aequorea victoria* green fluorescent protein [GFP; nucleotide sequences listed in Prasher et al., (1992) *Gene* 111:229] and the LacZ gene (produces  $\beta$ -galactosidase), both of which are well known in the art and may be obtained commercially.

A second aspect of the invention provides methods of forming eukaryotic cells for producing pseudotyped retroviruses. The method includes introducing into the cells described above the nucleotide sequences described above, i.e., those encoding the retroviral Gag, Pro and Pol polypeptides, and those encoding either a filoviral glycoprotein or at least two different viral glycoproteins, such as togaviral glycoproteins, into the cell.

The nucleotide sequences may be introduced into the desired cell utilizing a variety of vectors known to the skilled artisan. For example, plasmid vectors, cosmid vectors, and other viral vectors, such as retroviral



vectors, may be used. It is preferred that the nucleotide sequences encoding the Gag, Pro and Pol polypeptides are on a separate vector than the nucleotide sequences encoding the viral glycoproteins.

In one mode of practicing the invention, plasmid vectors are  
5 advantageously used to introduce, or transfect, the nucleotide sequences into the selected cell. A wide variety of plasmid vectors may be used, including pTRE, pCMV-Script and pcDNA3, although pcDNA3 is a preferred vector. The *gag*, *pro* and *pol* nucleotide sequences are preferably on the same plasmid, and, as discussed above, are preferably contiguous  
10 to each other. However, the skilled artisan is aware that other spatial configurations of the nucleotide sequences may be utilized when constructing the plasmids. The vector also preferably includes a promoter 5' to, or upstream from, the *gag* nucleotide sequence. The vectors may further include other regulatory elements, such as enhancer sequences, as  
15 discussed above.

The nucleotide sequences encoding the viral glycoproteins are preferably on a separate plasmid, or other vector, than the *gag*, *pro* and *pol* nucleotide sequences. The viral glycoprotein encoding sequences, such as the sequences encoding either the filoviral glycoproteins or those  
20 encoding at least two different viral glycoproteins (such as togaviral glycoproteins) are also preferably operably linked to a promoter sequence described above. It is also understood that the nucleotide sequences encoding at least two different viral glycoproteins may be arranged on a vector such that the nucleotide sequences encoding one of the  
25 glycoproteins are present on one vector and the sequences encoding the other glycoprotein are present on a different vector. It is preferred, however, that such sequences are on the same vector, and preferably contiguous to each other so they will be transcribed utilizing the same promoter. In one preferred form of the invention, the promoter sequence is  
30 a cytomegalovirus promoter sequence. Plasmids, or other vectors carrying the nucleotide sequences encoding the viral glycoproteins, may

also include other regulatory elements, such as enhancers, as described above.

The vectors may be introduced into the cells in a variety of ways known to the skilled artisan, for example, discussed in *Current Protocols in Molecular Biology*, John Wiley and Sons, edited by Ausubel et al. (1988) and Maniatis, et al., *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory (1989). For example, vectors may be transfected into a cell by a calcium phosphate precipitation method. Other methods for introduction of the vectors include, for example, electroporation and lipofection.

The nucleotide sequences may be introduced into the cells by a transient transfection procedure such that the proteins encoded by the respective sequences will be produced in a transient fashion as described above. By introducing the MMLV gene sequences and the E<sub>2</sub>-E<sub>1</sub> coding region from the Ross River virus (RRV) described above into a cell, we have determined that the cell lines produce pseudotyped retrovirus for a period of about 48 hours. However, it is preferred that the sequences are stably introduced. That is, it is preferred the nucleotide sequences become integrated into chromosomes of the cells into which they are introduced. In this way, the cells will stably produce pseudotyped retrovirus for a longer period of time compared to the transient expression. As used herein, a "stable cell line" or "stable cell" is defined as one that has chromosomally-integrated the nucleotide sequences described above and can produce pseudotyped retrovirus indefinitely (i.e., for the life span of the cell).

Furthermore, in order to form such stable cells, it is necessary to use selectable markers to screen for cells which have chromosomally-integrated the introduced DNA. Accordingly, the plasmid vectors, or other vectors, into which the respective nucleotide sequences are cloned may include such selectable markers.

A wide variety of selectable markers may be used. Typical selectable markers allow growth of only those cells which have been

transfected or transduced and thereby stably produce a desired protein. Examples of selectable markers that may be used include antibiotic resistance genes, including the neomycin gene, the hygromycin phosphotransferase gene and the bleomycin resistance gene which confer resistance to G418, hygromycin and zeocin, respectively. Other selectable markers include, for example, mutant mouse dihydrofolate reductase gene (confers resistance to methotrexate), and the bacterial gpt gene (selects for cells that can grow in a medium containing mycophenolic acid, xanthin and aminopterin). These selectable markers are discussed in *Retroviruses*, Cold Spring Harbor Laboratory Press, p. 444, edited by Coffin, J.M., Hughes, S.H. and Varmus, H.E. (1997).

In many cases, one may wish to quickly visually detect those cells which have taken up a vector and that produce a specified protein from the vector. Visually detectable components, or markers, include the *Aequorea victoria* green fluorescent protein as discussed above. When forming a cell that includes a visually detectable component, or marker, the nucleotide sequences encoding the marker may also be introduced into the cell as described above. For example, the nucleotide sequence encoding the green fluorescent protein may be placed in a recombinant MMLV genome or in a plasmid (to form plasmid MFG.S-GFP) by methods known to the art. For example, plasmid MFG.S-GFP may be formed by including in plasmid MFG [produced by methods known in the art and as exemplified by Ory et al., *PNAS USA*, 93:11400-11406 (1996)] the nucleotide sequence encoding the green fluorescent protein, surrounded by the nucleotide sequences described above, such as LTRs and the psi sequence. Cells that have taken up the vector and express the nucleotide sequences encoding a protein may be identified and separated from cells that do not express the sequences by a fluorescence-activated cell sorting procedure as known in the art. A visually detectable marker may also be formed from reaction of  $\beta$ -galactosidase (produced by the LacZ gene) with a substrate, such as X-gal.

Moreover, when growing cells that produce inventive pseudotyped retroviruses, the cells should be grown to no more than about 50% confluency, more preferably no more than about 25% confluency, and the pH of the culture medium should be maintained at about 7 by the frequent  
5 changing of culture medium. These conditions are conducive for production of cells that stably produce the pseudotyped retroviruses and should be strictly followed.

In a third aspect of the present invention, pseudotyped retroviruses that include viral glycoproteins (as discussed above) disposed in their lipid  
10 bilayer are provided. In one embodiment, at least two different viral glycoproteins are present in the lipid bilayer, such as togaviral glycoproteins. In alternative embodiments the glycoprotein is a filoviral glycoprotein.

In one embodiment, such pseudotyped retroviruses include a core  
15 RNA genome that is surrounded by, or enclosed within, a viral capsid. The genome preferably includes a nucleotide sequence encoding a protein selected to be subsequently produced by a cell. The genome further includes other nucleotide sequences for formation of the pseudotyped retrovirus, such as 5' and 3' LTR sequences that are operably linked to the  
20 nucleotide sequence encoding the desired protein as described above. Reverse transcriptase and integrase are also enclosed within the capsid, which gives the retrovirus the ability to incorporate a gene encoding a desired protein into a genome of a cell after the retrovirus contacts, or is incubated with, the cell. For example, the pseudotyped retrovirus may be  
25 used to incorporate a gene encoding an enzyme in a host cell that is incapable of producing the enzyme, or produces a non-functional enzyme as discussed above. Other sequences known to the art that are useful for transducing genes may also be present in the RNA genome.

The pseudotyped retrovirus may include other proteins, in addition to  
30 integrase, that aid its stable integration into the chromosomes of a target

cell. For example, with respect to a lentivirus, the pseudotyped retrovirus may include proteins such as vpr, vif and vpu.

In yet other preferred embodiments, the pseudotyped retrovirus may include a nucleotide sequence encoding a visually detectable component, or marker, such as *Aequorea victoria* green fluorescent protein as discussed above. Such a retrovirus may be advantageously used in a method of determining viral entry into a cell discussed above. Moreover, such a virus is advantageously used in the methods of the present invention to ensure that the pseudotyped retroviruses that are formed are replication incompetent (i.e., do not have all the sequences necessary in their viral genome to produce progeny retroviruses). For example, supernatant isolated from cells transduced by the vectors and contacted with a test cell should not result in localization of the fluorescent protein in the test cell.

In a fourth aspect of the present invention, methods of introducing nucleotide sequences into a cell are provided. In one embodiment, the method includes contacting, or transducing, a cell permissive for either filoviral entry, or entry of a virus having at least two different viral glycoproteins in its lipid bilayer such as a togavirus, with a retrovirus that has been pseudotyped with a filoviral glycoprotein or at least two different viral glycoproteins, such as togaviral glycoproteins, as described above that includes the desired nucleotide sequence in its genome. When the nucleotide sequences encode a desired protein, the cell is selected so that it also preferably allows expression of the selected nucleotide sequence. The level of transduction may be obtained by assaying methods known to the skilled artisan, and include assaying for the protein of interest encoded by the introduced nucleotide sequences or assaying for the presence of the nucleotide sequences. Viruses having at least two different viral glycoproteins in their lipid bilayer have a broad host range. For example, as togaviruses are pantropic (i.e., can invade, or infect, many different cell types with no special affinity for any particular cell type), a wide variety of

permissive cell types well known to the art may be chosen for use in the method, including for example, skin cells, muscle cells, fibroblasts, fat cells and central nervous system cells.

Other viruses having at least two viral glycoproteins in their lipid bilayer include those previously described above. Cells permissive for these viruses are well known to the skilled artisan. Similarly, as filoviruses infect a broad range of cells, a wide variety of cells known to the art that are permissive for filovirus entry may also be selected, including, for example, kidney cells, liver cells, muscle cells and fibroblasts.

In a fifth aspect of the present invention, methods of screening agents effective in blocking viral entry into a cell are provided. The methods allow for direct screening as the viral entry step can be detected in the method. If such agents were tested with a wild type virus, for example, multiple rounds of replication may occur and steps other than viral entry may thus be affected (e.g., such as replication of RNA, production of proteins, etc.). In such a case, one would not know if the agent affects the entry step or some other, indirect step. Thus, the present method allows for direct quantitation of viral entry as compared to remote quantitation.

In one embodiment of the methods of the present invention, a method includes (a) treating a pseudotyped retrovirus having a retroviral capsid, a lipid bilayer that surrounds the retroviral capsid, at least two different viral glycoproteins disposed in its lipid bilayer and a nucleotide sequence encoding a marker, preferably a visually detectable marker (or one that is capable of visual detection as described above) that is enclosed within the retroviral capsid, with an agent effective in blocking entry into a cell of the virus having at least two different viral glycoproteins in its lipid bilayer to form a treated pseudotyped retrovirus; (b) treating a cell permissive for entry of a virus having at least two different viral glycoproteins in its lipid bilayer with the treated pseudotyped retrovirus; and (c) identifying cells having the desired marker. In one embodiment, the retrovirus may have togaviral glycoproteins disposed in its lipid bilayer, and

the cells are permissive for togaviral entry. In alternative embodiments, the retrovirus may have a filoviral glycoprotein, such as a Marburg virus glycoprotein, disposed in its lipid bilayer, wherein the cells that are treated are permissive for Marburg virus entry.

5           Cells that are advantageously used in a method of screening agents effective in blocking viral entry into a cell are those that are permissive for entry of the specific virus, and will therefore depend on the virus used. Cells permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer are the same as recited in the  
10   method of introducing nucleotide sequences into a cell as discussed above. Similarly, cells permissive for Marburg virus entry include those described above used in the method of introducing nucleotide sequences into a cell. If it is not known whether a cell is permissive for viral entry, this can readily be determined by the skilled artisan using routine procedures. One way of  
15   determining whether a cell is permissive for viral entry is to transduce the cell with a pseudotyped retrovirus of the present method encoding a marker, and cells that have the marker may be identified by methods known to the art. The marker may be a visually detectable marker, such as the green fluorescent protein or  $\beta$ -galactosidase (i.e., one that gives rise to  
20   a visually detectable marker) described above. The selected cell should also allow for expression of the gene products encoded and carried on the viral genome

          A wide variety of agents may advantageously be screened in the present invention, including, immunological agents such as monoclonal  
25   and/or polyclonal antibodies. For example, monoclonal antibodies or polyclonal antisera against E<sub>2</sub>, or other viral glycoproteins, may advantageously be used. Various pharmacological agents may also be screened in the present method in the same way, and include proteins, peptides or various chemical agents.

30           In one preferred method, the vector, in (a) above, is treated, or incubated with, the agent for a time period sufficient for interaction of the

agent with the viral glycoprotein. Although this time period may vary depending on the nature of the agent and the viral glycoprotein, agents effective in blocking viral entry tend to effectively interact with the glycoprotein in a period of about 10 to about 60 minutes.

5           In (b), the cell is incubated, or contacted, with the treated pseudotyped retrovirus for a time period sufficient for viral entry. This time period may vary, depending on the specific cell type chosen and the specific viral glycoprotein present in the lipid bilayer of the pseudotyped retrovirus as the skilled artisan knows. However, the time period can  
10 typically range from about 1 to about 6 hours, but is typically about 1 to about 2 hours.

Cells having the desired marker may be identified in (c) by observing the presence of the marker. Any of the visually detectable markers previously described above may be utilized in the method. However, a  
15 preferred marker is the *Aequorea victoria* green fluorescent protein. Cells into which this marker has been introduced may be identified and separated from cells without the marker (cells not transduced by the retrovirus) by fluorescence-activated cell sorting as described above.

Furthermore, yet another embodiment of a method of screening  
20 agents effective in blocking viral entry into a cell includes (1) treating a cell permissive for entry of a virus having at least two different viral glycoproteins in its lipid bilayer with the agent to form a treated cell; (2) contacting the treated cell with a pseudotyped retrovirus having a retroviral capsid, a lipid bilayer that surrounds the retroviral capsid, at least two  
25 different viral glycoproteins disposed in its lipid bilayer and a nucleotide sequence encoding a marker, preferably a visually detectable marker (or one that is capable of visual detection as described above), that is enclosed within the retroviral capsid; and (3) identifying cells having the desired marker. As above, the retrovirus may have togaviral glycoproteins  
30 disposed in its lipid bilayer, and the cells are permissive for togaviral entry. In alternative embodiments, the retrovirus may have a filoviral glycoprotein,



such as a Marburg virus glycoprotein, disposed in its lipid bilayer, wherein the cells that are treated are permissive for Marburg virus entry. The cells and agents advantageously used in this embodiment are the same as described in the previous embodiment.

5           In this alternative embodiment, the cells in (1) above are treated, or incubated with, the agent for a time period sufficient for interaction of the agent with the cell to form a treated cell. Although this time period may vary depending on the nature of the agent and the cell, agents effective in blocking viral entry tend to effectively interact with the cell in a period of  
10       about 1 hour.

          In (2), the treated cell is incubated, or contacted, with the pseudotyped retrovirus for a time period sufficient for viral entry. The time period may vary, depending on the specific cell type chosen and the specific viral glycoprotein in the lipid bilayer of the pseudotyped retrovirus  
15       as the skilled artisan knows. However, the time period ranges from about about 1 to about 6 hours, but is typically about 1 to about 2 hours.

          Cells having the desired marker may be identified in (3) by the same method as described in (c) of the previous embodiment.

          In a sixth aspect of the present invention, kits for forming inventive  
20       pseudotyped retroviruses are provided. The kits include a first nucleotide sequence encoding a retroviral Gag polypeptide, a second nucleotide sequence encoding a retroviral Pro polypeptide, a third nucleotide sequence encoding a retroviral Pol polypeptide and a fourth nucleotide sequence encoding at least one viral glycoprotein. In one embodiment of  
25       the invention, the fourth nucleotide sequence encodes at least two different viral glycoproteins, such as togaviral glycoproteins and preferably alphaviral glycoproteins. In alternative embodiments, the fourth nucleotide sequence encodes a filoviral glycoprotein, preferably a Marburg virus glycoprotein. The sequences and methods of obtaining such sequences  
30       are discussed above. In general, the kits include sterile packaging which secures the various kit components in spaced relation from one another

sufficient to prevent breakage of the components during handling of the kit. For example, it is a common practice to utilize molded plastic articles having multiple compartments or areas for holding the kit components in spaced relation.

5           The inventive pseudotyped retrovirus are further useful in methods of identifying cell surface receptors that allow viral entry. In one embodiment, an inventive pseudotyped retrovirus may be employed in a method that identifies cell surface receptors for a virus having at least two different viral glycoproteins disposed in its lipid bilayer. The method  
10 includes (a) constructing a cDNA library from a first cell that is permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer; (b) transfecting a second cell with a cDNA-carrying vector wherein the second cell is non-permissive or semi-permissive for entry of a pseudotyped retrovirus that includes a retroviral capsid, a lipid bilayer  
15 wherein the lipid bilayer surrounds the retroviral capsid, at least two different viral glycoproteins disposed in its lipid bilayer and a nucleotide sequence encoding a desired marker wherein the nucleotide sequence is enclosed within the retroviral capsid; (c) transducing the second cell with the pseudotyped retrovirus; (d) identifying cells having the marker; and (e)  
20 identifying the cDNA insert in the transduced cell. In alternative embodiments, the cDNA library is constructed from a first cell permissive for entry of a Marburg virus and the second cell is transduced with a retrovirus pseudotyped with the Marburg virus glycoprotein.

          In a preferred method, the first cell is permissive for togaviral entry,  
25 further preferably alphaviral entry, and the second cell is transduced with a retrovirus pseudotyped with togaviral glycoproteins, preferably alphaviral glycoproteins.

          In (a), a cDNA library may be constructed by methods well known to the skilled artisan as described in *Current Protocols in Molecular Biology*,  
30 John Wiley and Sons, edited by Ausubel et al. (1988). For example, mRNA may be isolated from the first cell by breaking the cell membrane and

extracting and purifying the mRNA by known methods. The mRNA may be used as a template to form cDNA, which may then be cloned into various vectors as described above, such as plasmid vectors, by use of various restriction enzymes and DNA ligase as known in the art. Bacterial cells, or  
5 other similar cells, may be transfected with the expression vectors to form the cDNA library.

The first cell may be chosen from the cells permissive for entry of a virus having at least two different viral glycoproteins disposed in its lipid bilayer, such as an alphavirus, or a virus having a filoviral glycoprotein  
10 disposed in its lipid bilayer, such as a Marburg virus glycoprotein, or other filovirus glycoprotein as described above.

In (c), the second cell may be transduced with a pseudotyped retrovirus having a nucleotide sequence encoding a desired marker as described above in the embodiment described above of the method for  
15 screening agents effective in blocking viral entry into a cell and in (d), the transduced cells may be identified by methods discussed above, such as fluorescence activated cell sorting.

The second cell may be selected from non-permissive cells, preferably mammalian, known in the art. For example, in the case of the  
20 pseudotyped retrovirus that includes at least two viral glycoproteins disposed in its lipid bilayer, such as those from the Ross River virus, non-permissive cells include chicken embryo fibroblasts. One skilled in the art may also determine what other cells are non-permissive for alphaviruses, such as the Ross River virus, and the filoviruses, such as Marburg or  
25 Ebola virus, by the methods described herein as well as other methods known to the art.

The cDNA insert in the transduced eukaryotic cell may be identified and recovered by known methods, including amplifying known sequences in the cDNA-containing plasmids by PCR.

30 Reference will now be made to specific examples illustrating the compositions and methods above. It is to be understood that the examples

are provided to illustrate preferred embodiments and that no limitation to the scope of the invention is intended thereby.

## EXAMPLE 1

### Cells and Cell Culture

5 E86nlslacZ cells, Baby Hamster Kidney (BHK) cells, and mouse NIH3T3 fibroblasts were grown in Dulbecco's Modified Eagle Media (D-MEM, Sigma) with 10% Calf Serum (Gibco-BRL), 0.1 mg/ml streptomycin (Sigma) and 10 U/ml penicillin (Sigma)(D-MEM CS/PS). E86nlslacZ cells  
10 are NIH 3T3 cells that express MMLV capsid proteins, produced as known in the art and as described in Taylor, G.M. and Sanders, D.A. (1999) *Mol. Biol. of the Cell* (1999), in press, were constructed by stably transfecting GP+E86 cells of Markowitz et al. (1988) *J. of Virol.* 62:1120-1124 with MFG.S-nlslacZ. MFG.S-nlsLacZ is a retroviral vector encoding a nuclear  
15 localized  $\beta$ -galactosidase activity, produced as known in the art and as described in Ory, et al. (1996) *PNAS USA* 93:11400-11406.

Human HeLa,  $\Phi$ NX cells, gpGFP and gpnlslacZ cells were grown in D-MEM FBS/PS).  $\Phi$ NX packaging cells are second generation human embryonic kidney 293T cells transfected with MMLV *gag* and *pol* genes as  
20 described in Grignani et al. (1998) *Cancer Res.*, 58:14-19 and Pear et al., (1993) *PNAS USA*, 90:8392-8396. gpGFP cells are obtained by transfecting  $\Phi$ NX cells with retroviral vector MFG.S-GFP-S65T, a retroviral vector encoding the *Aequorea victoria* green fluorescent protein S65T mutant as described in Taylor, G.M. and Sanders, D.A. (1999) *Mol. Biol. of*  
25 *the Cell* (1999), in press. gpGFP cells therefore produce envelope-deficient replication- incompetent MMLV particles carrying MFG.S-GFP-S65T. gpnlslacZ cells were developed in our laboratory by cotransfecting MFG.S-nlsLacZ and pJ6 $\Omega$ puro [constructed as described in Morgenstern and Land (1990), *Nucleic Acids Res.*, 18:1068] into  $\Phi$ NX cells, growing  
30 transfected cells in D-MEM FBS/PS supplemented with 2  $\mu$ g/ml puromycin (Sigma) and antibiotic-resistant colonies were isolated and screened for the

production of high-titer replication-incompetent virus resulting from transient transfection with penv1min, a vector that encodes the wild type MMLV envelope protein [as described in Taylor, G.M. and Sanders, D.A. (1999) *Mol. Biol. of the Cell* (1999), in press].

5 VSV-G pseudotyped retrovirus-producing 293GPGnslacZ cells, constructed as described in Ory, et al. (1996) *PNAS USA* 93:11400-11406, were grown in D-MEM FBS/PS supplemented with 2 µg/ml puromycin and 1 µg/ml tetracycline (Sigma). As expression of the VSV-G protein in these cells is repressed by the presence of tetracycline in the medium, forty-eight  
10 hours before collection of pseudotyped virus the medium in which the 293GPGnslacZ cells were grown was replaced with D-MEM FBS/PS.

All cells were grown at 37°C and under 5% CO<sub>2</sub>. Moreover, the cells were grown at a density of no more than about 50% confluency and the medium was changed at intervals sufficient to maintain the pH of the  
15 medium at about 7.

## EXAMPLE 2

### Generation of Cell Lines Transiently Producing RRV-MMLV Pseudotyped Retrovirus

20

#### RRV Glycoprotein Expression Plasmid Construction

The region encoding the Ross River virus envelope glycoproteins was amplified from pRR64, a plasmid which contains the full-length Ross River viral genome [full-length sequence described in Faragher et al.,  
25 (1988), *Virology* 163:509-526] as described in Kuhn et al. (1991), *Virology* 182:430-441, by the polymerase chain reaction using Pfu polymerase and two primers complementary to the viral genome at nucleotides 8375-8386 (5'-CGGGATCCACCATGTCTGCCGCGCT-3') and 11312-11330 (5'-CGCTCTAGATTACCGACGCATTGTTATG-3') [the amplified sequences  
30 from plasmid pRR64 are shown in SEQ ID 1, beginning at nucleotide 3, and an additional "at" sequence (nucleotides 1 and 2 of SEQ ID 1) was

added at the 5' end of the pRR64 sequence]. The amplified fragment, which contained the RRV E<sub>3</sub>-E<sub>2</sub>-6K-E<sub>1</sub> coding region, was digested with the restriction endonucleases Bam HI and XbaI and ligated into BamHI and XbaI sites of pBacPac, a Baculovirus expression vector available from Clontech. The resulting plasmid was digested with BamHI and XbaI, and the fragment containing the RRV E<sub>3</sub>-E<sub>2</sub>-6K-E<sub>1</sub> coding region was ligated into the BamHI and XbaI sites in the pcDNA3 mammalian expression vector available from Invitrogen. The resulting plasmid was designated pRRV-E<sub>2</sub>-E<sub>1</sub>. SEQ ID 1 also shows the amino acid sequence of the E<sub>3</sub>-E<sub>2</sub>-6K-E<sub>1</sub> polypeptide.

#### Transient Transfection Procedure

In preparation for transfection,  $0.5 \times 10^6$   $\Phi$ NX cells, or gpnIslacZ cells, were washed with PBS (137 mM NaCl, 27 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.47 mM K<sub>2</sub>HPO<sub>4</sub>, pH 7.4) prior to incubation with 2 ml Opti-MEM (Gibco-BRL) for 30 minutes at 37°C in a 5% CO<sub>2</sub> atmosphere. 2 µg of pRRV-E<sub>2</sub>-E<sub>1</sub> and 2 µg of MFG.S-GFP-S65T was incubated with 300 µl Opti-MEM and 24 µl lipofectAMINE™ (Gibco-BRL) for 30 minutes at room temperature prior to dilution with 2.4 ml Opti-MEM. The resulting mixture was incubated with the cells for seven hours at 37°C in a 5% CO<sub>2</sub> atmosphere. Medium was replaced with DMEM FBS/PS for a further 48-hour incubation at 37°C in a 5% CO<sub>2</sub> atmosphere before collection of the supernatant medium for analysis of the transduction capacity of and level of glycoprotein incorporation into viral particles. When the gpnIslacZ cells were transfected, a similar protocol was followed except that the transfected DNA consisted solely of 4 µg of pRRV-E<sub>2</sub>-E<sub>1</sub>.

#### Transduction by Recombinant Retroviruses

HeLa, BHK and NIH 3T3 cells were transduced by the following method. Supernatant medium from recombinant-virus-producing cells was filtered through a 0.45 µm filter, mixed with hexadimethrine bromide

(Sigma) (final concentration 8 µg/ml) and incubated with cells for five hours at 37°C in a 5% CO<sub>2</sub> atmosphere. The recombinant virus-containing medium was then replaced with D-MEM CS/PS medium. Cells transduced with MFG.S-GFP-S65T were washed 48 hours after infection with PBS, then lifted from the plate with PBS containing 1 mM EDTA. The cells were then analyzed with a Coulter XL-MCL Flow Cytometer using a 525 nm band-pass and a 488 nm air-cooled argon laser. The level of glycoprotein incorporation into viral particles was determined by Western blotting as described in Example 4.

#### Analysis

Cell have been constructed that produce infectious pseudotyped virus containing the glycoproteins from the Ross River virus. The titer of virus was found to be  $1 \times 10^3$  TU/ml supernatant. The cells were able to produce the pseudotyped retrovirus for a period of 48 hours. As MMLV only infects mouse cells such as NIH 3T3 and the Ross River virus glycoprotein-pseudotyped retrovirus is able to infect NIH 3T3 cells, as well as BHK (hamster) and HeLa (human) cells, it has clearly been demonstrated that the host range of these retroviruses is increased by incorporation of the Ross River glycoproteins in the virus.

This example also shows that at least two different viral glycoproteins, each having a different membrane-spanning domain, can be incorporated into a retroviral particle (i.e., a retrovirus).

### EXAMPLE 3

## Generation of Stable Cell Lines Producing RRV-MMLV Pseudotyped Retrovirus

### Stable Transfection Procedure

5           0.5 x 10<sup>6</sup>  $\Phi$ NX or gpnIslacZ cells were transfected following the protocol in Example 2, except that the DNA that was transferred was only 8  $\mu$ g of pRRV-E<sub>2</sub>-E<sub>1</sub> and 0.4 $\mu$ g of plasmid pJ6 $\Omega$ puro coding for puromycin resistance [Morgenstern and Land, *Nucleic Acids Res.* 18, 1068 (1990)] and the DNA mixture contained 48  $\mu$ l lipofectAMINE<sup>TM</sup> and 600  $\mu$ l Optim-  
10 MEM. Selection with medium containing 2  $\mu$ g/mL puromycin began at 48 hours post-transformation. Clonal colonies of cells were isolated after two weeks of selection. The resulting cell lines derived from the  $\Phi$ NX cells were designated SafeRR and the resulting cell lines derived from the gpnIslacZ cells were designated SafeRRnIslacZ.

15           Titer was measured by infection of NIH3T3 cells as described below. Infection occurred in the presence of 8  $\mu$ g/ml polybrene and infectious supernatant was changed to media without polybrene 5 hours post-infection.

### 20   Transduction by Recombinant Retroviruses

          The same protocol of Example 2 was followed, except that forty-eight hours after the infection, the cells transduced with virus bearing MFG.S-nIslacZ were fixed with 0.5% glutaraldehyde (Sigma) and then incubated with 1 mg/ml of the  $\beta$ -galactosidase detection reagent 5-bromo-  
25 4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal, Fisher) in a staining buffer (1 mM MgCl<sub>2</sub>, 50 mM K<sub>3</sub>Fe(CN)<sub>6</sub> and 50 mM K<sub>4</sub>Fe(CN)<sub>6</sub>) for 3 hours prior to the determination of the proportion of blue cells as provided in Sanes, et al. (1986), *Embo J.*, 5:3133-3142.

### 30   Analysis

          Cells that permanently produce the above pseudotyped retroviruses have been constructed. SafeRR cells were found to produce pseudotyped



retrovirus at a titer of  $1 \times 10^3$  TU/ml supernatant. SafeRR-nslacZ cells were found to produce pseudotyped retrovirus at a titer of about  $1 \times 10^5$  TU/ml supernatant. SafeRR cells may be advantageous in introducing desired nucleotide sequences into a cell. Another advantage is that the expression of the Ross River virus glycoproteins are not toxic to the cells.

#### EXAMPLE 4

##### Immunodetection of Incorporation of RRV-E<sub>2</sub> into Pseudotyped Retrovirus Produced by SafeRR-nslacZ cells

This example demonstrates that the recombinant retrovirus contains the Ross River glycoproteins.

Supernatant medium from a 10 cm tissue-culture dish of confluent SafeRRnslacZ cells (described in Example 3), or precursor gpnlacZ cells (described in Example 1), was passed through a 0.45  $\mu$ m filter and spun through a 30% sucrose cushion at 25K rpm for 2.5 hours in a Beckman 50.2 titanium rotor. Material collected through the centrifugation was suspended in SDS-PAGE buffer (0.05% bromophenol blue, 0.0625 M Tris-HCl pH 6.8, 1% SDS, 10% glycerol). Cell lysates were prepared by washing cells with 10 ml PBS followed by 2 ml cell lysis buffer (50 mM Tris-HCL, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100). Aliquots of total lysed material were mixed with SDS-PAGE buffer and analyzed electrophoretically. PAGE-separated proteins were transferred to nitrocellulose membranes at 44 mA for 2 hours in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes were blocked with 5% powdered milk in washing solution (20 mM Tris-HCl, pH7.6, 137 mM NaCl, 0.1% Tween-20). Blocked membranes were reacted with pAbE2 (anti-Ross River E<sub>2</sub> rabbit polyclonal antiserum; provided by Richard Kuhn and produced by methods known to the art) at a 1:5000 dilution for two hours and goat anti-rabbit Horseradish Peroxidase (HRP)-linked secondary antibody (Chemicon, 1 mg/ml) at a 1:5000 dilution for thirty minutes.

Western blots were visualized with Enhanced Chemiluminescent Reagents (Amersham Pharmacia Biotech) by methods known in the art.

### Analysis

5           In order to clarify that E<sub>2</sub>-E<sub>1</sub> were incorporated into the MMLV particles and could be mediating the infection observed in Example 3, both virus producing cells and infectious supernatants were analyzed by SDS-PAGE and Western blotting with a polyclonal E<sub>2</sub> antiserum.

          As seen in FIG. 1, a 50kDa and a 60kDa immunoreactive protein  
10       were present in a lysate of SafeRR-nlslacZ (express RRV E<sub>2</sub>-E<sub>1</sub> pseudotyped MMLV). These are appropriate masses for E<sub>2</sub> and unprocessed E<sub>2</sub>-E<sub>3</sub>. Western analysis of virus collected from infectious supernatant revealed only the fully processed 50 kDa protein.

15

## **EXAMPLE 5**

### **Formation of Syncytia in Stable SafeRR-nlslacZ Cell Lines at Acidic pH**

20

          This example shows that SafeRR-nlslacZ cell lines are capable of forming syncytia at acidic pH, implying that entry of alphavirus into cells is dependent on the low pH environment normally found in endosomes.

25       SafeRR-nlslacZ or  $\Phi$ NX cells, obtained as described in Examples 3 and 1, respectively, were grown to near confluence, washed with PBS and treated with fusion buffer [PBS containing 10 mM 2-(N-morpholino)ethane sulfonic acid and 10 mM HEPES adjusted to pH 5.5] for one minute. The low pH solution was replaced with D-MEM FBS/PS, then cells were  
30       incubated in a CO<sub>2</sub> incubator at 37°C, and the cells were stained with Giemsa solution 5 hours after treatment and photographed.

### Analysis

          As seen in FIG. 2A, syncytia are detectable. No syncytia were observed in the treated  $\Phi$ NX cells that are shown in FIG. 2B. It is seen in

FIG. 2C that syncytia are also not detected when the SafeRR-nlslacZ cells are incubated in pH 7 fusion buffer. These results, indicating that Ross River virus glycoprotein-promoted membrane fusion is triggered by an acidic medium, are consistent with the data obtained by other laboratories that indicate the entry of alphavirus is dependent upon the low pH environment normally found in endosomes [other data discussed in Strauss and Strauss, (1994) *Microbiol. Rev.*, 58:491-562].

### EXAMPLE 6

#### Effect of Lysosomotropic Weak Bases on Infection By RRV-MMLV Pseudotyped Retrovirus

This example shows that the RRV-MMLV pseudotyped retrovirus enters cells through an endocytic pathway.

NIH 3T3 cells were pretreated for one hour with various concentrations of ammonium chloride or chloroquine in PBS as seen in FIG. 3. Medium containing  $1.5 \times 10^5$  TU/ml of supernatant of either wild type MMLV, VSV-G pseudotyped retrovirus or Ross-River E<sub>2</sub>-E<sub>1</sub> pseudotyped retrovirus (produced by SafeRRnlslacZ cells) containing various concentrations of bases (as seen in FIG. 3) as well as 8 µg/ml polybrene was incubated with the cells in a CO<sub>2</sub> incubator at 37°C. The virus-containing medium was replaced with D-MEM CS/PS 6 hours after infection. The cells were stained with a β-galactosidase detection reagent (X-gal) at 48 hours post infection, and blue cells were counted. The results are shown in FIG. 3.

#### Analysis

Ammonium chloride and chloroquine inhibit the acidification of endosomes and inhibit cellular entry of viruses that are taken up by endocytosis and that require exposure to low pH for virus-cell membrane fusion to occur as reported in Marsh and Helenius, *Adv. Virus Res.* (1989), 36:107-151. MMLV entry is known not to involve low pH-induced virus-cell

membrane fusion and infection by VSV-G pseudotyped retrovirus is known to involve low pH-induced virus-cell membrane fusion. These retroviruses therefore served as controls. The results show that chloroquine only partially affects wild type MMLV entry as seen in FIG. 3A, and that both  
5 chloroquine and ammonium chloride inhibit VSV-G pseudotyped retrovirus entry. It can therefore be concluded that the dramatic inhibition of transduction by Ross River glycoprotein-pseudotyped viruses in the presence of ammonium chloride and chloroquine is a direct effect upon entry, as all of the macromolecules required for the other necessary  
10 processes (viral uncoating, reverse transcription, integration, etc.) are identical with those contained in the relatively uninhibited MMLV-Env-bearing viruses. This example illustrates one of the advantages of the inventive pseudotype system of the present invention; the effects of an experimental manipulation on viral entry into a cell may be specifically  
15 investigated independent of any effects on other steps in replication.

### EXAMPLE 7

#### Neutralization of MMLV Pseudotyped with RRV E<sub>2</sub>-E<sub>1</sub> Coding Region

20 This example shows that retroviruses pseudotyped with the Ross River virus E<sub>2</sub>-E<sub>1</sub> are inhibited from entering a cell when pre-incubated with antibodies against E<sub>2</sub>.

Supernatants from SafeRR-nlsLacZ or wild type MMLVnlsLacZ (MMLV that includes RNA encoding  $\beta$ -galactosidase and the *env* gene  
25 proteins) producing cells were incubated with dilutions of Ross River virus monoclonal 10C9 [produced as described in Smith, (1995) *PNAS USA* 92:10648-10652] in ascites fluid or dilutions of Ross River virus polyclonal (pAbE<sub>2</sub>) antiserum (provided by Richard Kuhn and produced by methods known to the art) prior to infection of NIH3T3 cells. No significant inhibition  
30 of infectivity was observed in wild type MMLVnlsLacZ while a 60% inhibition of infectivity of RRV-MMLVnlsLacZ was observed at a 1:500

dilution of polyclonal antiserum. Inhibition was most significant with monoclonal 10C9, which binds to the cell receptor binding region on RRV E<sub>2</sub> (Smith et al., *Proc Natl Acad Sci USA* 92, 10648-10652 (1995)). For example, a 70% inhibition of infectivity was observed in supernatant from  
5 SafeRR-nlslacZ cells with a 1:500 dilution of ascites fluid containing monoclonal 10C9.

10

### EXAMPLE 8

#### **Generation of Cell Lines Transiently Producing Ebola-MMLV Pseudotyped Retrovirus Including Nucleotide Sequences Encoding GFP in its Genome**

15 This example shows production of cell lines that transiently produce MMLV pseudotyped with Ebola-Zaire glycoprotein.

pEZGP1 was produced by cloning into the polylinker of plasmid pcDNA3 nucleotide sequences corresponding to nucleotides 6029-8253 [sequences 6029-8253, corresponding to nucleotides 132-2354 described  
20 in Genbank as Accession Number U23187, are shown in SEQ ID 2 from the Ebola Zaire virus genome, with the exception that an additional "a" has been inserted between nucleotides 1027 and 1028 in SEQ ID 2 compared to the Genbank sequence] from the complete Ebola Zaire genome [described in Sanchez, et al., (1993) *Virus Res.* 29(3):215-240] obtained  
25 by digestion of the MP1153 plasmid provided by Dr. Anthony Sanchez with Eco RI and HindIII. SEQ ID 2 also shows the amino acid sequence of the Ebola Zaire glycoprotein.

gpGFP cells were transiently transfected with pEZGP1 using lipofectAMINE™ (Gibco, BRL) and Opti-MEM media (Gibco, BRL). The  
30 gpGFP cells were plated at 5x 10<sup>5</sup> cells/60 mm plate 24 hours prior to transfection. The cells were washed and incubated for 30 minutes at 37°C with 2 ml of Opti-MEM media. The DNA-LipofectAMINE™-Opti-MEM mixture (4µg DNA, 24 µl lipofectAMINE™, and 300 µl Opti-MEM media)

was incubated for 30 minutes at 25°C. After the 30 minute incubations, 2.4 ml of Opti-MEM media was added to the DNA-lipofectAMINE™ mixture. The resulting solution was layered onto the gpGFP cells. Eight hours later, the transfection mixture was removed and the cells were incubated with DMEM FBS/PS for 40 hours. The supernatant medium was filtered through a 0.45 µm filter and then incubated with 1 x 10<sup>6</sup> NIH 3T3 cells in the presence of 8 µg/ml polybrene for 4 hours. The recombinant-virus-containing medium was then replaced with D-MEM CS/PS. Forty-eight hours later the cells were removed from the plate, suspended in 1xPBS containing 1 mM EDTA, and analyzed by flow cytometry with a Coulter XL-MCL Flow Cytometer, using a 525 nm band-pass filter and a 488 nm air-cooled argon laser.

#### Analysis

Cell have been constructed that produce infectious pseudotyped virus containing glycoproteins from the Ebola Zaire virus. The titer of virus was found to be 4.5 x 10<sup>4</sup> TU/ml of supernatant. The cells were able to produce the pseudotyped retrovirus for a period of about 24 hours.

### **EXAMPLE 9**

#### **Generation of Stable Cell Lines Producing Ebola-MMLV Pseudotyped Retrovirus**

gpGFP cells were stably transfected with pEZGP1. gpGFP cells were plated at 5x 10<sup>5</sup> cells/60 mm plate 24 hours prior to transfection. The cells were washed and incubated for 30 minutes at 37°C with 2 ml of Opti-MEM media. The DNA-LipofectAMINE™-Opti-MEM mixture (8 µg of mutant DNA, 0.4µg of pJ6Ωbleo, 48 µl lipofectAMINE™, and 300 µl Opti-MEM media) was incubated for 30 minutes at 25°C. After the 30 minute incubations, 2.4 ml of Opti-MEM media was added to the DNA-LipofectAMINE™ mixture. The resulting solution was layered onto the gpGFP cells. Eight hours later the transfection mixture was removed and

the cells were incubated with DMEM FBS/PS for 40 hours before transferring the cells to 10 cm plates at two different dilutions (1/10 and 1/100). The following day, the media was changed to D-MEM FBS/PS containing 200 µg/ml of Zeocin. Colonies appeared after two weeks and  
5 were picked for screening by an infectivity assay described below. The cell lines so produced were labeled "SafeEbola-GFP".

The supernatant medium from the cells was filtered through a 0.45 µm filter and then incubated with  $1 \times 10^6$  NIH 3T3 cells in the presence of 8 µg/ml polybrene for 4 hours. The recombinant-virus-containing medium  
10 was then replaced with D-MEM CS/PS. Forty-eight hours later the cells were removed from the plate, suspended in 1xPBS containing 1 mM EDTA, and analyzed by flow cytometry with a Coulter XL-MCL Flow Cytometer, using a 525 nm band-pass filter and a 488 nm air-cooled argon laser.

Stable cell lines that produce pseudotyped retrovirus not containing  
15 specific nucleotide sequences such as those encoding the green fluorescent protein were produced in the same manner, except the parent cell line to the gpGFP cells were used instead (i.e., ΦNX cells, human embryonic kidney cells transfected only with MMLV *gag* and *pol* nucleotide sequences). These cell lines were labeled "SafeEbola".

20 As seen in FIG. 4, lower panel B, cells (45.8% as determined by fluorescence activated cell sorting) transduced with pseudotyped retroviruses produced from SafeEbola-GFP cells exhibited detectable green fluorescence.

## 25 Analysis

Cell lines that stably produce MMLV virus pseudotyped with Ebola Zaire glycoprotein have been produced. The cells indefinitely produce the pseudotyped retrovirus. The glycoprotein used to form the pseudotyped retrovirus is not toxic. The cells require diligence in care (i.e., changing the  
30 media every two days) so that the pH does not drop and syncytia formation does not occur.

### EXAMPLE 10

#### Formation of Syncytia in Stable SafeEbola-GFP Cell Lines at Acidic pH

This example shows that SafeEbola-GFP cell lines are capable of forming syncytia at acidic pH.

5  $5 \times 10^5$  SafeEbola-GFP cells or  $\Phi$ NX cells, obtained as described in Examples 10 and 1, respectively, were plated on 60 mm tissue-culture dishes, grown to near confluence, washed with PBS and treated with fusion buffer [PBS containing 10 mM 2-(N-morpholino)ethane sulfonic acid and 10 mM HEPES adjusted to pH 5.5] for one minute. The low pH solution was replaced with D-MEM FBS/PS, incubated in a CO<sub>2</sub> incubator at 37°C, and the cells were stained with Giemsa solution 5 hours after treatment and photographed. As seen in FIG. 5A, the SafeEbola-GFP cell lines form syncytia at acidic pH, whereas no such syncytia are formed in  $\Phi$ NX cells as seen in FIG. 5B.

### EXAMPLE 11

#### Generation of Cell Lines Transiently Producing Marburg Virus Glycoprotein Pseudotyped Retrovirus

##### Marburg Glycoprotein Expression Plasmid

25 Marburg plasmid pMBGP1 was constructed from a plasmid from Hans-Dieter Klenk (Marburg, Germany). To construct this plasmid, the nucleotides 5931-8033 from the Marburg virus genome [the genomic nucleotide sequence HK Klenk, as delineated in Will et al. (1993), *J. Virol.* 67:1203-1210 and as seen in Genbank Accession Number Z12132 shown in SEQ ID 3] were cloned into the pSP72 plasmid (from Promega) under the control of the T7 promoter using Sall. The XhoI and Eco RI fragment of this plasmid was cloned into the XhoI and Eco RI polylinker sites of the



mammalian expression vector pcDNA3. SEQ ID 3 also shows the amino acid sequence of the Marburg virus glycoprotein.

#### Transient Transfection Procedure

5           The transient transfection protocol was identical to that recited in Example 8 (Ebola-glycoprotein transfection protocol), with the exception that, instead of pEZGP1, 4 µg of pMBGP1 was used.

#### Analysis

10           It has been shown that cell lines may be constructed that produce MMLV that is pseudotyped with the Marburg virus glycoprotein. The cell lines were found to produce the pseudotyped retroviruses at a titer of about  $1.4 \times 10^3$  TU/ml of supernatant. The cells were able to produce the virus for a period of about 24 hours. In data not shown, it was found that NIH  
15   3T3, BHK and HeLa cells can be efficiently transduced by this inventive pseudotyped retrovirus. This demonstrates the expanded host range of the pseudotyped retroviruses, which allows these pseudotyped retroviruses to be advantageously used to introduce desired nucleotide sequences into target cells.

20

### **EXAMPLE 12**

#### **Generation of Cell Lines Stably Producing Marburg Virus Glycoprotein Pseudotyped Retrovirus**

25

#### Stable Transfection Procedure

          The stable transfection protocol was identical to that recited in Example 9 (Ebola-glycoprotein transfection protocol), with the exception that 4 µg of pMBGP1 (described in Example 11) was used.

30

Analysis

It has been shown that cell lines may be constructed that stably, and thus indefinitely, produce MMLV that is pseudotyped with the Marburg virus glycoprotein. The cell lines were found to produce the pseudotyped retroviruses at a titer of about  $1.9 \times 10^3$  TU/ml of supernatant. The glycoprotein incorporated into the lipid bilayer of the pseudotyped retroviruses is not toxic. Moreover, the cells require diligence in care (i.e., changing of the media every two days) so that the pH does not drop and syncytia formation does not occur.

While the invention has been illustrated and described in detail in the drawings and foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiment has been shown and described and that all changes and modifications that come within the spirit of the invention are desired to be protected. In addition, all references cited herein are indicative of the level of skill in the art and are hereby incorporated by reference in their entirety.